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Primary Glioblastoma Cultures: Can Profiling of Stem Cell Markers Predict Radiotherapy Sensitivity?

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Running Title: Glioblastoma stem cell markers and radiotherapy sensitivity

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Abbreviations

ATP-binding cassette, sub-family G member 2, ABCG2; Aldehyde dehydrogenase 1, ALDH1; Basic fibroblast growth factor, bFGF; bone morphogenetic protein 2, BMP-2; bromodeoxyuridine, BrdU; cancer stem cell, CSC; Enhancer of zeste homolog 2, EZH2; epidermal growth factor, EGF; fetal calf serum, FCS; glial fibrillary acidic protein, GFAP; glioblastoma-initiating cell, GIC; leukemia inhibitory factor, LIF; L1 cell adhesion molecule, L1CAM; magnetic activated cell sorting, MACS; neural sphere cell medium, NSCM; Pleiomorphic adenoma gene-like 2, PLAGL2; SRY (sex determining region Y)-box 2, SOX2; standard error of the mean, SEM; serum-containing medium, SCM; serum-free medium, SFM; Tumor 269, T269

Abstract

Human glioblastomas may be hierarchically organized. Within this hierarchy glioblastoma-initiating cells (GIC) have been proposed to be more resistant to radiochemotherapy and responsible for recurrence. Here, established stem cell markers and stem cell attributed characteristics such as self-renewal capacity and tumorigenicity have been profiled in primary glioblastoma cultures to predict radiosensitivity.

Furthermore, the sensitivity to radiotherapy of different subpopulations within a single primary glioblastoma culture was analyzed by a flow cytometric approach using Nestin, SRY (sex determining region Y)-box 2 (SOX2) and glial fibrillary acidic protein (GFAP).

The protein expression of Nestin and SOX2 as well as the mRNA levels of Musashi1, L1CAM, CD133, Nestin and PLAGL2 inversely correlated with radioresistance in regard to the clonogenic potential. Only CD44 protein expression correlated positively with radioresistance. In terms of proliferation, Nestin protein expression and Musashi1, PLAGL2 and CD133 mRNA levels inversely correlated with radioresistance. Higher expression of stem cell markers does not correlate with resistance to radiochemotherapy in a TCGA (the cancer genome atlas) glioblastoma collective. SOX2 expressing subpopulations exist within single primary glioblastoma cultures. These subpopulations form predominantly the proliferative pool of the primary cultures and are sensitive to irradiation.

Thus, profiling of established stem cell markers revealed a surprising result. Except CD44, the tested stem cell markers showed an inverse correlation between expression and radioresistance.

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68 **Keywords:** CD133, glioma initiating cells, profiling, stem cell markers, radiotherapy

69 sensitivity, SOX2

Introduction

For many years, glioblastoma, the most frequent and aggressive primary brain tumor in adults, has been regarded as a clonal malignancy and was modelled *in vitro* by established glioma cell lines kept in serum-containing medium (SCM). Evidence emerged that primary glioma cell cultures may better reflect the genetic and biologic features of glioblastoma (Bjerkvig *et al.* 2005). At present, the search for glioblastoma subpopulations that are distinct from the main tumor and responsible for tumor initiation and progression as well as resistance to therapy is ongoing. Such cells are usually termed cancer stem cells (CSC) or glioblastoma-initiating cells (GIC), reflecting rather a concept than unequivocal evidence and only a few examples exist for these cells being a therapeutic target (Zhu *et al.* 2014). GICs are defined by their properties to self-renew, to express stem cell markers and most importantly to be highly tumorigenic and able to recapitulate a phenocopy of the tumor of origin in immunocompromized mice when transplanted orthotopically in low cell numbers.

We aimed at profiling different GIC cultures and different subgroups within single GIC cultures to evaluate how the various stem cell markers and techniques to detect stem cells, which are the product of an intense research over the last years (Bao *et al.* 2006; Bao *et al.* 2008; Fukaya *et al.* 2010; Rasper *et al.* 2010; Singh *et al.* 2003; Suva *et al.* 2009; Thon *et al.* 2010; Zheng *et al.* 2010) correlate with resistance to radiotherapy. Furthermore, we tried to detect the stem cell subpopulation within single GIC cultures, to analyse whether they form the radioresistant pool of the GIC culture.

The analysis was done on different levels. The clonogenic potential of the different GIC cultures was estimated by the limiting dilution assay (LDA), proliferation was measured by BrdU- or EdU-uptake. Radioresistance was assessed by the relative decline in proliferation and clonogenic potential after irradiation with 4 Gy. These results were correlated with the protein expression of the stem cell markers CD133,

CD15, CD44, Nestin and SOX2 measured by flow cytometry as well as the mRNA expression levels of various stem cell markers.

To confirm our data the same markers were analyzed in a radiochemotherapy-treated TCGA collective of glioblastoma patients. Finally, we evaluated with the help of flow cytometry whether stem-cell subpopulations exist within a single GIC culture which behave differentially from the non-stem cells. Exemplarily, the *in vitro* data were verified *in vivo* by orthotopical xenotransplantation of glioma initiating cells in nude mice.

Material and methods

Cell culture

Tumor samples were obtained from adult patients diagnosed with glioblastoma after informed consent. Glioma-initiating cell cultures (GIC cultures) were established from freshly dissected tumor tissue with a success rate of 1/4 tumors. Tumor and neurosphere cultures were cultured as described (Hemmati *et al.* 2003). Cells were seeded in neural sphere cell medium (NSCM) containing DMEM:F12 medium enriched with B27 supplement, basic fibroblast growth factor (bFGF) (20 ng/ml), epidermal growth factor (EGF) (20 ng/ml) and leukemia inhibitory factor (LIF) (20 ng/ml) (Hemmati *et al.* 2003). To propagate cells in culture they were split mechanically. For all the experiments requiring a single cell suspension spheres were split with accutase (PromoCell, Heidelberg, Germany). To obtain adherent, differentiated cells, tumor cells were cultured in SCM medium (10% FCS, fetal calf serum) on poly-L-lysine-coated tissue flasks for 15 days. Alternatively, differentiation was induced by withdrawal of EGF and bFGF, culture on a poly-L-lysine coated surface and supplementation of recombinant bone morphogenetic protein 2, BMP-2, (10-50 ng/ml) or CNTF (50 ng/ml), (R&D systems, Minneapolis, MN, USA) with and without 5-Azacytidin (3 μ M) (AXXORA, Lausen Switzerland) 3 days prior to fixation and subsequently analyzed by immunofluorescence microscopy and flow cytometry (Lee *et al.* 2008). Glioblastoma origin was confirmed by comparative genomic hybridization (Toedt *et al.* 2011) indicating typical genomic alterations on chromosomes 7q, 10p, 17q (Holland *et al.* 2010). A subset of the GIC cultures (WJ and PJ) was analyzed by Illumina Human Methylation 450 array performed from DNA extracted from cell lines. The array data was used to calculate a low-resolution copy number profile as previously described (Sturm *et al.* 2012).

Human astrocytes (ScienCell Research Laboratories, Carlsbad, CA, USA) were kept in astrocyte medium (ScienCell Research).

Quantitative Real-Time PCR (qRT-PCR)

Total RNA was extracted using a RNA purification system (Qiagen, Hilden, Germany) and treated with RNase-free DNase I to remove genomic DNA (Roche, Mannheim, Germany). cDNA was prepared from 5 µg of total RNA using the Superscript RNase H–Reverse Transcriptase (Invitrogen, Karlsruhe, Germany) and random hexamers (Sigma-Aldrich, Taufkirchen, Germany). For qRT-PCR, gene expression was measured in an ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) with SYBR Green Master Mix (Eurogentec, Cologne, Germany) and primers at optimized concentrations (Opitz *et al.* 2009). Primers (Sigma-Aldrich, Taufkirchen, Germany) were selected to span exon–exon junctions if possible. Standard curves were generated for each gene and the amplification was 90–100% efficient. Relative quantification of gene expression was determined by comparison of threshold values. All results were normalized to GAPDH. The sequences for the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and the genes evaluated were as follows:

GAPDH_fw: CTCTCTGCTCCTCCTGTTGAC

GAPDH_rev: TGAGCGATGTGGCTCGGCT

CD133_fw: CATCCACAGATGCTCCTAAGGC

CD133_rev: AAGAGAATGCCAATGGGTCCA

ALDH1_fw: TGCTTCCGAGAGGGGGCGAC

ALDH1_rev: TCCATTGTCGCCAGCAGCAGAC

ABCG2_fw: ACGAACGGATTAACAGGGTCA

ABCG2_rev: CTCCAGACACACCACGGAT

156 L1CAM_fw: GGTCCCTGGAGAGTGACAACGAGGA
 157 L1CAM_rev: GGCCCCTGAGCTGTCATTGCC
 158 Musashi1_fw: GGGAGGTGAAGGAGTGTCTG
 159 Musashi1_rev: CTGGTCCATGAAAGTGACGAA
 160 EZH2_fw: GCTCAAGAGGTTTCAGACGAG
 161 EZH2_rev: GCTGTTTCCATTCTTGGTTTAAG
 162 PLAGL2_fw: GAGTCAAGTGAAGTGCCAATGT
 163 PLAGL_rev: TGAGGGCAGCTATATGGTCTC
 164 Nestin_fw: GGTGGCCACGTACAGGACCCT
 165 Nestin_rev: AGATCCAAGACGCCGGCCCT

166

167 **Clonogenic capacity and sensitivity towards radiotherapy**

168 To assess the clonogenic capacity limiting dilution assay, LDA, was performed as
 169 described (Eirew *et al.* 2010). Shortly, cells were dissociated with accutase.
 170 Afterwards, 24 wells of a 96-well microwell plate were each plated with 300, 50, 8
 171 and 1 cells in 0.2 ml of NSCM. After three weeks microwell plates were analyzed for
 172 wells showing clones and clonal frequency as well as the standard error of the mean
 173 (SEM) was calculated with L-Calc free online software (STEMCELL Technologies).
 174 Stem cell frequency was expressed by 1 / the minimum amount of cells necessary to
 175 form a colony. To evaluate the radioresistance LDA was also performed after
 176 irradiation at 4 Gray (Gy).
 177 Experiments to assess differential radiosensitivity were also performed within
 178 subfractions of GIC cultures kept in NSCM and analyzed by flow cytometry.
 179 Proliferation assessed by bromodeoxyuridine, BrdU, incorporation and cell cycle
 180 distribution (DNA content) of different GIC subfractions were measured 72 h after
 181 irradiation at 4 and 8 Gy.

182

183 **Immunocytochemistry and flow cytometry**

184 Immunocytochemistry of neurosphere cultures was performed as described
185 (Geschwind *et al.* 2001). Neurospheres were fixed in ice-cold methanol or in 4%
186 paraformaldehyde and immunostained with rabbit anti-Nestin (1:200; Chemicon,
187 Temecula, CA, USA), mouse anti- TuJ1 (1:100; Chemicon), rabbit anti-gial fibrillary
188 acidic protein (GFAP; 1:500; Chemicon) followed by Alexa fluorophore-conjugated
189 secondary antibodies (1:100; Molecular Probes, Karlsruhe, Germany).

190 Furthermore, CD133 and CD15 expression were evaluated by flow cytometry with
191 anti-CD133/1-Phycoerythrin (PE) or CD133/2-PE antibody and CD15-Viogreen
192 (Miltenyi Biotec, Bergisch-Gladbach, Germany). CD44, SOX2, Nestin expression and
193 the amount of cycling cells were evaluated using flow cytometry after staining with
194 anti-CD44-Alexa700, anti-human SOX2-PE, anti-human Nestin-647 and anti-Ki67-
195 Alexa647 antibodies purchased from BD-Biosciences. Nuclei were counterstained
196 with 4,6- diamidino-2-phenylindole, DAPI, and 7-aminoactinomycin, 7-AAD.

197 To assess proliferation cells were incubated for 3-12 h with 10 μ M BrdU or EdU at
198 37°C and 5% CO₂. Afterwards the cells were fixed and stained using the BrdU-Flow
199 and EdU-Flow Kit from BD Biosciences (San Jose, CA, USA).

200 Cells were analyzed in a BD-FACS Canto II flow cytometer, final data were
201 processed with the help of FlowJo flow cytometry analysis software. To translate flow
202 cytometric expression data for statistic evaluation the mean fluorescence value after
203 staining with the respectively isotype antibody was log-transformed and subtracted
204 from the log-transformed mean fluorescence value of the specific antibody. This
205 transformation translates the shift seen when performing overlays in graphically
206 presented flow data. Values smaller than 0.12 corresponding to a specific

fluorescence index, SFI, (Hueber *et al.* 2003) smaller than 1.3 were regarded as no specific staining for a marker and therefore no marker expression.

Animal experiments and preparation of mouse brains for histology

All animal work was performed in accordance with the German animal protection law (Approving institution: Regierungspräsidium Karlsruhe). Tumorigenicity was determined by injecting 50 to 10^5 glioblastoma-derived neurosphere cells suspended in PBS orthotopically into the right striatum of 6-12 weeks old athymic female mice (CD1 nu/nu, Charles River, Sulzfeld, Germany) by a stereotactic procedure. To reduce pain animals were anesthetized with xylazine and ketamine. Neurological symptoms were assessed daily. Symptomatic animals were rapidly sacrificed to prevent pain. The brains of the sacrificed animals were recultured as described for the fresh human tumor specimens. Recultured cells were reimplanted at 50-1000 cells after new spheres had formed to assess whether cells could be serially transplanted. Cryostat transverse brain sections (8 μ m) were stained with hematoxylin-eosin (H&E) or with anti-human Nestin antibody (Chemicon International, Billerica, USA) and analyzed by AxioVision software (Carl Zeiss, Jena, Germany). To evaluate the *in vivo* sensitivity towards irradiation, 2×10^5 GIC were orthotopically implanted in five animals per group. Seven days later cerebral irradiation (6 Gy) was performed in the experimental groups. For local irradiation, brains of nude mice were irradiated using electrons from a standard Linac radiation source. Positioning and shielding of the animals were achieved by a lead/plastic device that allows the exact application of the radiation with a 90% isodose to the targeted 7x 7 mm brain section, sparing the throat of the mice (Tabatabai *et al.* 2006).

TCGA collective

To assess the influence of mRNA expression of defined stem cell markers on survival in isocitrate dehydrogenase (*IDH*) wild type patients, methylation (Illumina HumanMethylation27 BeadChip, n=294 samples and Illumina HumanMethylation450 BeadChip, n=126 samples), mRNA expression (z-score transformed) and clinical data were obtained from the database of The Cancer Genome Atlas (TCGA, <http://cancergenome.nih.gov>) and from cBioPortal (Cerami *et al.* 2012) at Jan 15 2013.

To detect the gliomas CpG island methylator phenotype (G-CIMP) (and exclude G-CIMP+ patients from further analysis), unsupervised hierarchical clustering of methylation data was performed as described previously (Wiestler *et al.* 2013). Briefly, probes (i) targeting the X and Y chromosomes, (ii) containing a single nucleotide polymorphism within 5 base pairs of and including the CpG site and (iii) not mapping uniquely to the human reference genome (hg19), allowing for one mismatch, were removed. The 1500 (Illumina HumanMethylation27 BeadChip) and 8000 (Illumina HumanMethylation450 BeadChip) most variable (by SD) probes were kept and unsupervised hierarchical clustering was performed for each platform.

Patients (n = 134) included in this analysis were (i) treatment-naïve at the time of tissue extraction and (ii) initially treated with radio- and chemotherapy (temozolomide) as in the EORTC-26981-22981 trial (Stupp *et al.* 2005). Disease-free survival as reported by the TCGA was the primary endpoint. Statistics: The association of mRNA expression and disease-free survival was assessed through univariate Cox proportional hazards regression models. Univariable p values were adjusted for multiple testing using Benjamini-Hochberg correction in order to control the false discovery rate (Benjamini Y 1995). Analyses were carried out using R (Version 3.01) (R Development Core Team 2011).

259

260 **Statistical analysis**

261 Statistical significance was assessed by student's t-test (Excel, Microsoft, Seattle,
262 WA, USA) at $p < 0.05$ (significant) or $p < 0.01$ (highly significant). All *in vitro*
263 experiments reported here were performed at least two times, flow cytometry data
264 were repeated at least twice except tumor Ma-1 which stopped growing in cell
265 culture. For the assessment of a monotonic relationship between the expression
266 (both on protein and mRNA level) of defined stem cell factors and proliferation &
267 clonogenicity, Spearman's rank correlation test was used.

268

Results

Glioblastoma-initiating cells

Sphere forming cultures were established from freshly dissected tumor tissue, which was cultivated after dissociation in neurosphere medium. All GICs formed neurospheres under this condition (Fig. 1A).

Exposure to serum-containing medium (SCM) for 15 days or BMP-2 in neurosphere medium (NSCM) without growth factors for 3 days led to differentiation of the sphere cultures measured by up-regulation of GFAP and the neuronal marker β III-tubulin as shown by immunofluorescence microscopy and flow cytometry for a better quantification (Fig. 1B-D, Suppl. Fig.1). These differentiation approaches neither abrogated the expression of the neural stem cell marker Nestin nor the progenitor and stem cell marker SOX2 as observed before (Gursel *et al.* 2011).

Cells cultured in neurosphere medium formed orthotopic brain tumors at low cell numbers. As few as 50 cells / mouse brain were enough to form deeply infiltrating tumors mimicking the growth pattern of human glioblastomas (Fig. 1E and Suppl. Fig.1D+E). The time of tumor formation varied from ~90 to more than 200 days in the different GIC-cultures after implantation of thousand cells (Suppl. Fig.1E). Interestingly, differentiation in SCM for 15 days did not abrogate tumorigenicity (Suppl. Fig.1D+E).

Finally, serial repassaging after explantation of the tumors, which had formed in CD1 nu/nu mice, again resulted in gliomas. This detailed analysis was performed for the GIC-cultures shown in Tab.1. The data presented and the data published before (Lemke *et al.* 2012) provide evidence that the cells cultured exhibit stem cell characteristics.

Comparative genomic hybridization (CGH) analysis was performed for T1, T325, T269, WJ, PJ, ZH161, ZH305, KNG002 and S24 and demonstrated the glioblastoma origin of the GIC-cultures examined (exemplarily in Suppl. Fig.2-4).

Stem cell marker profiling helped to predict the radiosensitivity of GIC-cultures in the limiting dilution assay (LDA)

As a high clonogenic potential by itself is supposed to be a characteristic stem cell feature (Bjerkvig *et al.* 2005), we performed LDA of 10 different GIC-cultures without and after irradiation at 4 Gy. The clonogenic potential expressed by one divided by the amount of cells necessary to form at least one new colony varied from 0.0115 in tumor MM to 0,88 in S24. In other words, between different GIC cultures 1.1 (S24) to 86 (MM) cells were necessary to form one new colony (Fig.2, upper part). We then evaluated the radioresistance of the different GIC cultures by dividing the amount of cells necessary to form at least one colony after irradiation with 4 Gy by the number of cells necessary without irradiation. In tumor KNG002 it took nearly 80x more cells after irradiation in the LDA to form at least one new colony while in tumor T325 only 1.5x the amount of cells were necessary (Fig.2, upper part). Tumors with a stronger reduction of the clonogenic potential after irradiation were regarded as more radiosensitive compared with the tumors where the difference was smaller. Interestingly, the clonogenic potential without therapy did not predict sensitivity to irradiation at 4 Gy.

In a next step, different stem cell markers were profiled in the GIC cultures to examine whether stem cell marker expression correlates with radioresistance in the LDA. Flow cytometric expression analysis was performed of the stem cell markers SOX2, Nestin, CD133, CD15 and CD44 in the ten GIC cultures evaluated in the LDA before. With the help of Spearman's rank correlation test we could show that SOX2

and Nestin expression inversely correlate while CD44 expression positively correlates with radioresistance in the LDA ($p \geq 0,05$) (Fig. 2 lower left).

To gain a broader look on more stem cell markers and assess whether qPCR analysis suffices to predict radioresistance we further analyzed the mRNA levels of CD133, Nestin, ALDH1 (Aldehyde dehydrogenase 1), ABCG2 (ATP-binding cassette, sub-family G member 2), L1CAM (L1 cell adhesion molecule), Musashi1, EZH2 (Enhancer of zeste homolog 2) and PLAGL2 (Pleiomorphic adenoma gene-like 2) in 9 of the GIC cultures examined in the LDA before.

Statistical analysis revealed that CD133, Nestin, Musashi1, PLAGL2 and L1CAM inversely correlated with radioresistance in the LDA.

Stem cell marker profiling can help to predict the radiosensitivity of GIC-cultures proliferation

We next evaluated the proliferation rate without and with 4 Gy irradiation of GIC cultures to examine another parameter, which might reflect radioresistance *in vitro*. Therefore, GIC cultures were treated for 6 hours with EdU and incorporation was measured by flow cytometry (Fig. 3, upper part). During this time tumor MM showed the weakest EdU-uptake in only ~13 % of the population while tumor PJ had the strongest proliferation rate with in EdU-uptake in ~41 % of the cells. After irradiation we could observe a general decline of the proliferation rate. Tumor 325 which was regarded as a very radioresistant tumor showed a relative proliferation of ~ 94 % after irradiation while radiosensitive tumors such as T1 and KNG002 went down to ~19 % of their proliferation rate without irradiation.

We did not observe a correlation between proliferation of the unsorted GICs and increased susceptibility to radiotherapy (Fig. 3; upper part). Aiming again to examine whether radioresistance in regard to the proliferation rate was predictable by stem

cell marker profiling, we correlated the relative proliferation rate at 4 Gy with the expression of the afore mentioned stem cell markers in flow cytometry and qPCR. On the protein level Nestin inversely correlated with radioresistance (Fig. 3; lower left), on the mRNA level Musashi1, PLAGL2 and CD133 expression showed an inverse correlation with radioresistance (Fig. 3; lower right).

***In vitro* radiosensitivity correlates with response to radiotherapy in a xenograft model**

Mounting evidence suggests that tumor stem cells occupy a special perivascular and/or perinecrotic niche. This niche is supposed to be necessary for GIC to maintain their stem cell phenotype (Ricci-Vitiani *et al.* 2010; Seidel *et al.* 2010; Seoane 2010; Hambardzumyan *et al.* 2008b). To strengthen the *in vitro* data on radiosensitivity, we performed an *in vivo* experiment allowing the cells to grow in an orthotopic environment. Exemplarily, T269 which belonged to the group of the radiosensitive tumors in the LDA and proliferation assay and the radioresistant T325 were implanted each at 2×10^5 cells in five CD1 nu/nu mice *per* group. T269-bearing mice became symptomatic 71 days after implantation. In contrast, animals orthotopically implanted with T325 cells became symptomatic around day 192. Interestingly, cranial irradiation with 6 Gy prolonged survival for 36 days in the T269 model, whereas the survival difference between irradiated and untreated animals implanted with T325 cells was not significant (Fig. 4). We concluded that the *in vitro* data were predictive for the *in vivo* response to irradiation.

Stem cell marker expression does not correlate with radiochemoresistance in the TCGA data base

To translate our data to a larger cohort which better reflects the high genetic heterogeneity of glioblastoma and its four defined subclasses (Verhaak *et al.* 2010) we re-evaluated our results in the TCGA glioblastoma patients collective. The influence of mRNA expression of the defined stem cell markers on disease-free survival of radiochemotherapy treated patients with *IDH* wild-type glioblastomas was assessed. Methylation, mRNA expression and clinical data were obtained from the database of The Cancer Genome Atlas (TCGA, <http://cancergenome.nih.gov>) and from cBioPortal. G-CIMP positive patients were excluded from further analysis to have a more homogeneous group of primary glioblastomas. Patients (n = 134) included in this analysis were treatment-naïve at the time of tissue extraction and initially treated with radio- and chemotherapy as in the EORTC 26981/22981 trial (Stupp *et al.* 2005). Disease-free survival as reported by the TCGA was the primary endpoint. This analysis could not demonstrate a correlation between the examined stem cell markers and survival of the patients (Tab. 2).

Evaluation of GIC culture subpopulation based on intracellular marker expression

So far, the experiments performed demonstrated that higher expression of stem cell markers correlated inversely with radioresistance, with the exception of CD44. Since glioblastomas are highly heterogeneous tumors and a lot more factors interfere with radiotherapy such as p53-status and expression of checkpoint kinases, we were interested to see whether there are subpopulations within a single GIC culture showing a differential response to radiotherapy. Stem cells are supposed to divide asymmetrically which should allow identifying different subpopulations within GIC cultures (Chen *et al.* 2010; Lathia *et al.* 2011). Hence, GIC cultures might be composed of more and less differentiated cells that might be masked by mere

expression analysis of the whole population. As CD133, CD44 and CD15 were only be detected in subset of the GIC cultures, we focused on intracellular stem cell markers.

Therefore, we looked at the expression of Nestin, a marker for neural stem and progenitor cells (Singh *et al.* 2004;Strojniak *et al.* 2007), which correlated inversely with radioresistance in the LDA and proliferation assay. The GIC cultures tested showed a monophasic Gaussian distribution of Nestin expression with a homogeneous BrdU-uptake leading us to the conclusion that cells with a higher Nestin expression did not proliferate better than low Nestin expressing cells. Furthermore all the GICs tested were nearly 100% positive for Nestin. Hence, Nestin did not seem suitable for distinguishing subfractions within individual GIC cultures (Suppl. Fig. 5). Likewise, the more differentiated astrocytic marker GFAP did not allow to differentiate subfractions within GIC cultures *in vitro* (data not shown).

Finally, we examined the expression of the transcription factor SOX2, a progenitor and stem cell marker, which demonstrated to be central for the tumorigenicity of GIC cultures. Studies in mice have implicated SOX2 as one of three factors in regulating pluripotency in embryonic stem cells (Fong *et al.* 2008;Gangemi *et al.* 2009;Ikushima *et al.* 2009). In contrast to non-neoplastic human astrocytes, SOX2-mRNA was expressed in GIC cultures (Fig. 5A) and could be detected in glioblastoma tissue sections (Fig. 5B). Interestingly, on the protein level GIC cultures tested partly formed subfraction with a SOX2 positive and a SOX2 negative fraction. T269 was nearly 100% positive for SOX2 expression while the SOX2^{positive} fraction was the smallest in T325 (Fig. 5C).

The SOX2 subpopulations mainly form the proliferating pool of GIC cultures and are sensitive to irradiation

Combining BrdU-incorporation analysis and SOX2-staining in flow cytometry revealed that SOX2^{positive} -cells better proliferated than SOX2^{negative}-cells. T269 e.g. demonstrated a maximal proliferating fraction of 29.5% of cells entering the S-phase within 6 hours. Of these 29.5 % proliferating cells. 28.8 % were SOX2^{positive} and 0.7% were SOX2^{negative} (Fig. 5c; left). The SOX2^{positive} fraction formed 95.6% of all cells in this tumor. T325 was composed of ~ 40 % SOX2^{positive}-cells. Again, the proliferating cells, 2,49% in this example, were in ~98% SOX2 positive (Fig. 5c; right).

To further characterize the SOX2^{positive} subpopulation, SOX2 and Ki67 were co-stained to assess the cycling cells. Here, ~64% of T325 expressed SOX2. 10.6% of the SOX2^{positive} cells were cycling detected by a positive Ki67 staining. Only 0,2% of the 36% SOX2^{negative} cells showed a weak positivity for Ki67 (Fig. 5D). We concluded that the cycling cells can be almost exclusively found in the SOX2^{positive} fraction.

To finally assess whether SOX2^{positive} cells are sensitive to irradiation, we irradiated five different GIC cultures with 4 and 8 Gy and analyzed the proliferation capacity 72 h after treatment. All 5 GICs treated showed a dose dependant reduction of the proliferation rate after irradiation (Fig. 6). T269 e.g. was composed of a nearly 100 % SOX2^{positive} fraction, of which ~26% took up BrdU within 6 h. After irradiation at 8 Gy T269 was still composed of nearly 100 % Sox2^{positive} cells but only ~ 6 % were still proliferating.

The SOX2^{positive} cells which formed predominantly the proliferating pool of the 5 GIC cultures were, sensitive to irradiation in terms of their proliferation capacity (Fig. 6). Irradiation did not reduce the amount of SOX2-positive cells 72 h after irradiation. SOX2^{negative} cells showed also a reduced proliferation rate after irradiation but they formed only a minority of the proliferating fraction.

Discussion

The aim of this study was to examine whether markers used to identify cancer stem cells are of value to predict radiosensitivity in primary glioma cultures obtained from patient biopsies. We were interested to find out whether profiling of stem cell characteristics and markers of different GIC cultures or profiling of stem cell markers within a single GIC culture would allow predicting radiosensitivity of different GIC cultures or a stem cell subgroup within a single GIC culture. So far cancer stem cells are regarded highly relevant for recurrence after treatment in glioblastoma patients (Bao *et al.* 2006;Beier *et al.* 2008;Hambardzumyan *et al.* 2008b;Hambardzumyan *et al.* 2008a).

First, we cultivated freshly dissected tumor tissue in neurosphere cell medium until sphere formation was observed. We performed several experiments demonstrating that the cultures fulfilled stem cell criteria and were of glioma origin. The GIC-cultures characterized in detail (Tab.1), formed tumors in low cell numbers, showed a multilineage differentiation capacity, were propagated in culture for many passages (except tumor Ma-1) and demonstrated a high clonogenic potential. Surprisingly, differentiation in serum-containing medium without growth factor supplementation did not abrogate the expression of the stem cell markers Nestin and SOX2 (Fig. 1, Supl, Fig. 1) nor the tumorigenic potential in several tumors (Suppl. Fig.1). We did not examine systematically whether the growth pattern, like the capacity to infiltrate, changed after the differentiation approach. The fact that tumors still formed argued against a terminal differentiation by the strategies applied. Similarly, Jiang *et al.* published in an experimental glioma model in neonatal Gtv-a Arf(-/-) mice induced by platelet-derived growth factor-B (PDGF-B) (Jiang *et al.* 2011) a lingering tumorigenicity of their sphere cultures after induction of differentiation with FBS. The authors interpreted that this was due to the high plasticity of their GIC-cultures.

We next characterized the clonogenic potential in ten GIC cultures with the LDA (Fig. 2). A high clonogenic potential of the unsorted GICs was not associated with a higher radioresistance in the LDA. We concluded that clonogenicity which by itself is related to stemness (Bjerkvig *et al.* 2005) does not allow to predict radioresistance. However, the relative reduction of the clonogenicity may be used as a parameter for radioresistance allowing comparing the different GIC cultures examined. As a second parameter to evaluate radioresistance, we determined the relative decline of the proliferation rate 72 h after irradiation with 4 Gy measured bei EdU incorporation. Correlating radioresistance in the LDA and proliferation assay with the protein expression of the stem cell markers CD133, CD15, CD44, Nestin and SOX2 as well as the mRNA levels of Nestin, Musashi1, L1Cam, ABCG2, ALDH1, CD133, PLAGL2 and EZH2 revealed an unexpected result. Only CD44 protein expression correlated with radioresistance in the LDA, while Nestin and SOX2 protein expression as well as Nestin, L1CAM, Musashi1, PLAGL2 and CD133 mRNA levels correlated inversely with radioresistance in the LDA. The proliferation analysis showed a negative correlation for radioresistance and Nestin protein expression as well as Musashi1, PLAGL2 and CD133 mRNA-levels. The absolute proliferation rate of the different GIC cultures without irradiation was not predictive for radiotherapy sensitivity. We concluded that there is no trivial association between irradiation effects and proliferation. These unexpected results are in line with data published before by Beier *et al.* (Beier *et al.* 2008) who examined chemotherapy sensitivity in CD133 positive and negative cancer stem cell cultures. CD133 was originally suggested to be a key marker for GIC growing as spheroids (Lottaz *et al.* 2010;Singh *et al.* 2003;Singh *et al.* 2004). It is expressed in neural stem cells and has been attributed a role in the development of the central nervous system (Uchida *et al.* 2000). After the initial paper by Singh *et*

al. (Singh *et al.* 2003) several studies have identified stem cells by their expression of CD133. CD133 and related CD133^{positive} cells were associated with a phenotype of resistance towards radiotherapy (Bao *et al.* 2006;Rich 2007). Anyway, in the publication from Beier *et al.* CD133 negative cells were associated with chemoresistance. This is in line with a growing number of publications showing glioma initiation by CD133^{negative} cells that even gave rise to CD133^{positive} cells (Chen *et al.* 2010;Lee *et al.* 2006b;Wang *et al.* 2008) calling the importance of CD133 as a stem cell marker in question.

Our *in vitro* results, which allow predicting radiotherapy sensitivity, were strengthened by the *in vivo* experiment. Tumor T269, *in vitro* classified as a more radiosensitive tumor than T325, proved to be also more radiosensitive in orthotopic xenotransplanted nude mice treated at 6 Gy (Fig. 4).

Hence, the microenvironment, which is provided in the mouse, did not alter the differential *in vitro* radiosensitivity of the GIC lines. Considering that GICs are supposed to differentiate into endothelial cells and vessel like structures (Ricci-Vitiani *et al.* 2010) one should assume that GICs are capable to create their niche to some extent in the mouse brain. Therefore, we concluded that radiosensitivity measured *in vitro* by LDA and proliferation analysis mirrors the sensitivity of our GIC lines realistically and is more than an *in vitro* artefact. Although it is unclear whether cells implanted 7 days before irradiation as scheduled in the experiment are capable to establish an own niche for the stem cells. At least, the *in vivo* experiment which covers a time frame from more than 200 days underlined that the irradiation effects measured after 72h *in vitro* reflect more than a casual snap-shot.

To argue against a too small number of GIC cultures tested (n=10) we tried to translate our concept into a clinical context by correlating the mRNA level of accepted stem cell markers in a TCGA glioblastoma collective with their sensitivity to

radiochemotherapy measured by disease-free survival. Here, no stem cell marker tested predicted resistance or sensitivity to therapy (Tab. 2). There are several possible explanations for this lack of translation. The data in the TCGA are generated by tumor and bystander cells in an unknown ratio and *in vitro* culturing of GICs by itself is prone to amplify subtypes of glioblastomas (Laks *et al.* 2009). Thus, our experimental findings were not strengthened by this analysis. Yet, the widely accepted stem cell concept arguing that stem cell marker defined cells are more resistant and therefore the basis for recurrent disease was also not supported. An example for a more robust concept for *bona fide* stem cells might be given by stem cells driven by the nuclear receptor tailless (Tlx) (Zhu *et al.* 2014).

Finally, as glioblastomas are supposed to be hierarchically organized and stem cells divide asymmetrically, we established a multiparameter flow cytometric approach to examine cell cycle distribution and proliferation rate in different subfractions within a single GIC culture. We focussed on the intracellular markers Nestin and SOX2 trying to detect more radioresistant CSC subfractions. Nestin was not suitable to detect subpopulation *in vitro* within different GIC cultures since its expression was distributed evenly and all the GIC cultures tested were nearly 100% positive for Nestin (Suppl. Fig. 5). SOX2, on the other hand, differentiated subfractions within the GIC cultures tested. The amount of SOX2^{positive} cells varied from more than 95% to ~17% (Fig. 6). SOX2^{positive} cells almost exclusively expressed Ki67, which is a marker for cycling cells (Fig. 5D). But most importantly, the SOX2^{positive} proliferating subpopulation was sensitive to irradiation (Fig. 6). This was finally not surprising as we could demonstrate that the SOX2^{positive} cells formed mainly the proliferating fraction of the cells. Irradiation did not hamper SOX2 expression itself within the first 72 hours after irradiation but clearly reduced the amount of proliferating cells in five different GIC cultures within this subfraction.

550 These results explain why silencing of SOX2 attenuates tumorigenicity (Gangemi *et*
551 *al.* 2009) and is in line with the assumption that SOX2 cooperates with cyclin D1 in cell
552 cycle progression (Oppel *et al.* 2011).

553 The only positive correlation between stem cell marker expression and
554 radioresistance was detected for CD44 which is in line with published data showing
555 that CD44 promotes tumor cell resistance to reactive oxygen species-induced and
556 cytotoxic agent-induced stress by attenuating activation of the Hippo signalling
557 pathway (Xu *et al.* 2010).

558 To conclude, within the last decade the cancer stem cell concept has gained a lot of
559 attention in the glioma field. Many markers and techniques have been published to
560 detect the cancer stem cells within glioma samples. We tested some of these
561 markers and techniques to prove whether they are valuable to predict resistance to
562 radiotherapy, a feature, which is attributed to cancer stem cells. Our profiling
563 strategies revealed that the majority of the significantly correlating stem cell markers
564 tested show an inverse correlation with radioresistance except CD44. Kim *et al.*
565 came to a similar result when profiling samples of different radiochemotherapy
566 treated glioblastoma patients for CD133, CD15 and Nestin expression which did not
567 correlate with a better survival (Kim *et al.* 2011). Furthermore, SOX2 emerged to be a
568 marker useful to detect subfractions within single GIC cultures composing
569 predominantly the cycling and radiosensitive cells in our hands.

570 It is likely that cancer stem cells are not sufficiently characterized by single markers.
571 It is also likely that surface markers in solid tumors, analyzed after dissociation of the
572 tumor, do not represent a biology-related phenotype, but are prone to change by the
573 manipulation, may shift over time or in response to the microenvironment.
574 Considering further that we have achieved in only around 30-50% of all the patient-
575 derived tissue to establish a GIC culture it might well be that the *in vitro* data are

576 simply not appropriate to mirror the *in vivo* conditions. But taking all this limitations
577 into account and being aware of the fact that tumor stem cells derived from
578 glioblastomas cultured in bFGF and EGF are probably the best *in vitro* model we
579 have (Lee *et al.* 2006a) these data call the widely accepted theory that stem cells are
580 the source of therapy resistance in question. At least the markers published to detect
581 stem cells should not be used without scepticism. Finally, our data point at further
582 evaluating the role and therapeutic options of CD44 in radioresistance of
583 glioblastomas.

584

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The authors declare that they have no conflict of interest.

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Figure legends

Fig. 1: GIC cultures fulfill the stem cell characteristics sphere formation, multilineage differentiation and tumorigenicity

A: GIC cultures T325 and T269 form neurospheres in NSCM.

B: T269 cultured in SCM, serum-containing medium (**T269S**) and NSCM, neurosphere cell medium (**T269**). **T269S** shows upregulation of GFAP- and β III-tubulin expression, Nestin stays positive after induction of differentiation for 15 days in serum containing medium.

C: Differentiation can also be induced in T269 with bone morphogenic protein-2 (BMP-2) treatment for 72 h in NSCM devoid of EGF and FGF. β (III)-tubulin expression (Tuj1) was quantified by flow cytometry. It is induced after treatment with BMP-2 (dotted line: isotype-antibody; dense line: Tuj1-antibody, vertical dash: mean Tuj-1 expression of the undifferentiated NSCM-cultured cells,

D: BMP-2 can also induce the expression of the glial marker GFAP but does not reduce the expression of the stem cell markers Nestin and SOX2. Percentage of marker positive cells was quantified.

E: Tumor formation of T269 in nude mouse brain. Anti-human Nestin antibody (green) illustrates the infiltrative growth. Nuclei are counterstained with DAPI. Higher magnification in the lower panels of **E** demonstrate that T269 infiltrates far into the contralateral hemisphere.

Fig. 2: Stem cell marker expression correlates with radioresistance measured in the limiting dilution assay (LDA)

Upper part: Clonogenicity of 10 different GIC cultures is presented as 1/divided by the amount of cells necessary to form at least one new colony (small square points, black lines represent the standard error of the mean calculated by L-Calcul; STEMCELL technologies). Bars represent the relative clonogenicity after irradiation with 4 Gy calculated by dividing the amount of cells necessary to form one new colony at 0 Gy from the number at 4 Gy. High results represent more sensitive tumors. GICs were sorted along their radiosensitivity and colour coded. The most sensitive tumors are on the left side visualized in dark green, while the most resistant tumor can be found at the right side in dark red.

Lower part: The same 10 GIC cultures were analyzed for the expression of the signified stem cell markers by flow cytometry (left part) and 9 of the 10 GICs were further evaluated for the mRNA expression of several stem cell markers (right). The colours from dark green to dark red mirror the sensitivity to radiotherapy visualized in the upper part of the figure. Stars symbolize a significant inverse or positive correlation between a stem cell marker and radioresistance ($p \geq 0,05$).

Fig. 3: Stem cell marker expression correlates with radioresistance measured by proliferation

Upper part: The proliferation rate of 10 different GIC cultures is presented as the percentage of cells incorporating EdU within a pulse experiment of 6 h (small square points, black lines represent the standard deviation). Bars represent the relative proliferation rate in percent after irradiation at 4 Gy calculated by dividing the proliferating cells at 0 Gy from the number at 4 Gy. High results represent more resistant tumors. GICs were sorted along their radiosensitivity and colour coded. The most sensitive tumors are on the left side visualized in dark green, while the most resistant tumor can be found at the right side in dark red.

Lower part: The same 10 GIC cultures were analyzed for the expression of the signified stem cell markers by flow cytometry (left part) and 9 of the 10 GICs were further evaluated for the mRNA expression of several stem cell markers (right). The colours from dark green to dark red mirror the sensitivity to radiotherapy visualized in the upper part of the figure. Stars symbolize a significant inverse or positive correlation between a stem cell marker and radioresistance ($p \geq 0,05$).

Fig. 4: *In vivo* response to irradiation in orthotopically grown GLC tumors
underlines that T269 is more radiosensitive than T325

Kaplan-Meier survival analysis was performed for mice orthotopically implanted with 2×10^5 T269 or T325 cells (n=5) irradiated *in situ* (day 7 after implantation) at 6 Gy or not. Irradiation has only a significant therapeutic effect on mice implanted with T269 which survive approximately 36 days ~ 50%) longer. Mice implanted with T325 did not show a significant survival benefit.

Fig. 5: SOX2^{positive and negative} subpopulations exist in GIC cultures

A: quantitative RT-PCR demonstrates that GIC cultures express higher levels of SOX2 than human astrocytes (**hAS**) (*: $p < 0,05$; **: $p < 0,01$).

B: SOX2-staining of PFA-fixed human glioblastoma sample 1462 shows that tumor cells are heterogeneously positive for SOX2. Endothelial cells stained by CD31 do not express SOX2. Nuclei are counterstained with DAPI.

C: SOX2^{positive} cells form mainly the proliferating fraction of the GIC cultures T269, T1 and T325. The histogram was overlayed with the dot blot of T269 (left panel) to demonstrate how SOX2^{positive and negative} cells were gated (dashed line: isotype stained cells; dense line: SOX2 stained cells). SOX2 expression on the x-axis was plotted against BrdU-incorporation on the y-axis. Cells were treated for 6 h with BrdU.

D: SOX2^{positive} cells form almost exclusively the Ki67^{positive} cycling pool of T325. Double staining with Ki67 and SOX2 in flow cytometry demonstrates that practically all the cycling Ki67 positive cells belong to the SOX2^{positive} fraction (10.6%) while only 0.2% of the cycling cells are SOX2^{negative}.

Fig. 6: SOX2^{positive} cells form predominantly the proliferating pool of GIC cultures and are sensitive to irradiation

5 GIC cultures (T269, T325, T1, Ma-1, H1) were irradiated with 4 and 8 Gy *in vitro*. After 72h proliferation was measured by BrdU uptake for 6 h (y-axis) in the GIC cultures and plotted against SOX2 expression (x-axis) to allow differentiation between more stem cell related SOX2^{positive} cells and the SOX2^{negative} cells within one single GIC culture. All the GIC show a dose dependant decline of their proliferation rate after irradiation. The SOX2^{positive} cells, which formed predominantly the proliferating cells (control) are sensitive to irradiation. Irradiation does not lead uniformly to a change of the size of the SOX2 positive population 72 h after therapy.

Tab. 1: Comparison of GIC cultures T269, T1, T325 and Ma-1 in regard to established stem cell marker expression and functional stem cell characteristics

| GIC | Tumorigenicity 1000 cells | Tumor formation: Aggressiveness (days until symptomatic) | Infiltration | <i>In vivo</i> -passaging | Sphere formation | % of Nestin positive cells | % of Sox2 positive cells | CD 133 | longterm cell culture | Multilineage Differentiation | |
|-------------|------------------------------|--|--------------|---------------------------|------------------|----------------------------|--------------------------|--------|-----------------------|------------------------------|-------|
| | | | | | | | | | | neuronal | glial |
| T269 | 3/3 mice | 102 +/- 8.5 | ++ | + | + | 99% | 96-99% | + | + | + | + |
| T1 | 3/3 mice | 225 +/- 31.7 | + | + | + | 99% | 65-85% | - | + | + | + |
| Ma-1 | 3/3 mice | 87 +/- 4.7 | + | - | + | 99% | 80% | n.p. | - | n.p. | n.p. |
| T325 | 3/3 mice | 233 +/- 7.5 | + | + | + | 99% | 60-87% | - | + | + | + |

Comparison of tumorigenicity after orthotopic implantation of 10^3 cells, aggressiveness of tumor growth (assessed by the interval, in which the animals got symptomatic), infiltration and *in vivo* passaging capacity of tumor cells, multilineage differentiation and long-term cell culture, describing the capacity of a sphere culture to be passaged for more than ten times. The expression of SOX2, Nestin and CD133 was analyzed by flow cytometry. Tumor Ma-1, which grew very aggressively *in vivo* stopped proliferating *in vitro*. Hence, Ma-1 did not fulfil all cancer stem cell criteria (n.p.=not possible). (Stronger infiltration with tumor cells reaching the contralateral hemisphere was marked with ++).

Tab. 2: Stem cell marker expression does not correlate with radiochemo-resistance in a TCGA collective

| Gen | HR | 95% CI | p |
|---------------------|-------|--------------|-------|
| ABCG2 | 0,946 | 0,7328-1,22 | 0,963 |
| ALDH1A1 | 0,893 | 0,7064-1,128 | 0,963 |
| BMI1 | 0,962 | 0,7488-1,236 | 0,977 |
| EZH2 | 0,884 | 0,7081-1,104 | 0,963 |
| Integrin α 6 | 1,051 | 0,8408-1,315 | 0,963 |
| KLF4 | 1,087 | 0,8901-1,328 | 0,963 |
| Musashi1 | 0,835 | 0,5194-1,343 | 0,963 |
| NANOG | 0,997 | 0,8051-1,234 | 0,977 |
| NESTIN | 1,227 | 0,9185-1,638 | 0,963 |
| OLIG2 | 0,948 | 0,7819-1,149 | 0,963 |
| PLAGL2 | 1,091 | 0,9012-1,321 | 0,963 |
| Oct4 | 1,013 | 0,8073-1,272 | 0,977 |
| CD133 | 0,977 | 0,793-1,204 | 0,977 |

Stem cell marker mRNA expression of a TCGA collective of radiochemotherapy treated glioblastoma Patients (n=134) was correlated with the risk (Hazard Ratio, HR) to have a shorter disease-free survival. No marker shows a significant influence on disease-free survival after treatment (CI: Confidence interval, p: level of significance).

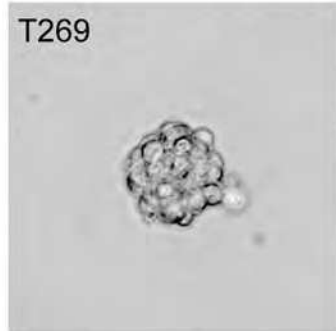


Fig. 1A

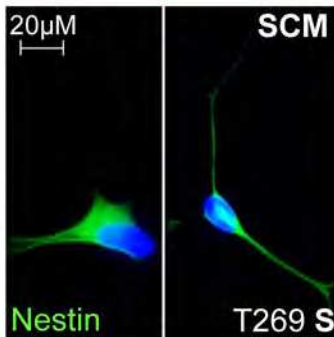
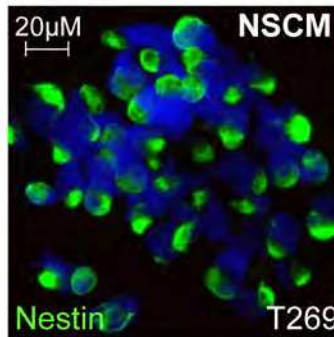
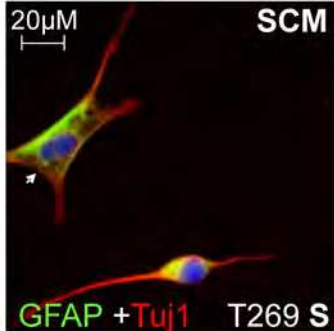
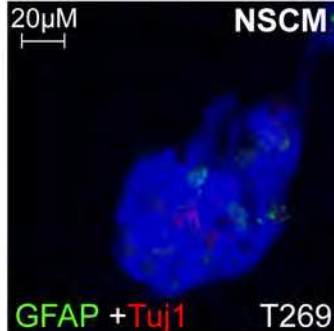


Fig. 1B

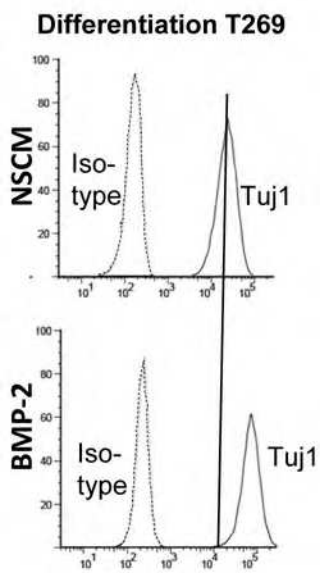


Fig. 1C

| | T269 NSCM | T269 BMP2 |
|----------|-----------|-----------|
| GFAP % | 72.1 | 97.5 |
| SOX2 % | 99.7 | 98.9 |
| Nestin % | 99.1 | 99.2 |

Fig. 1D

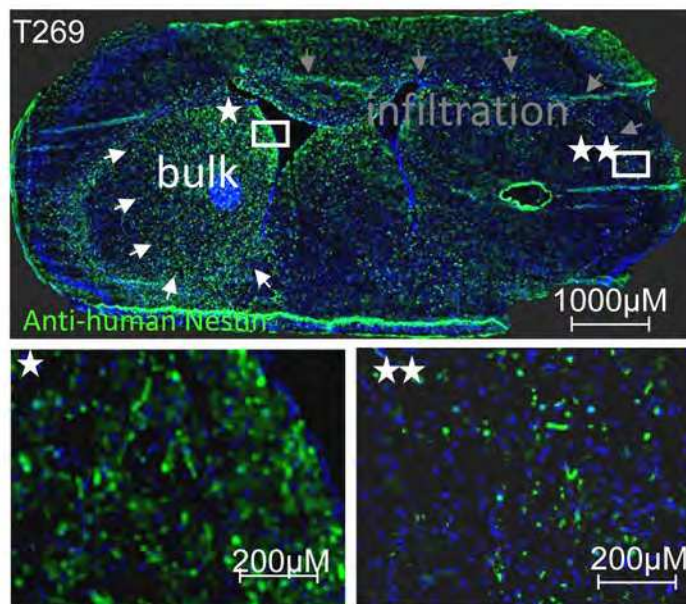
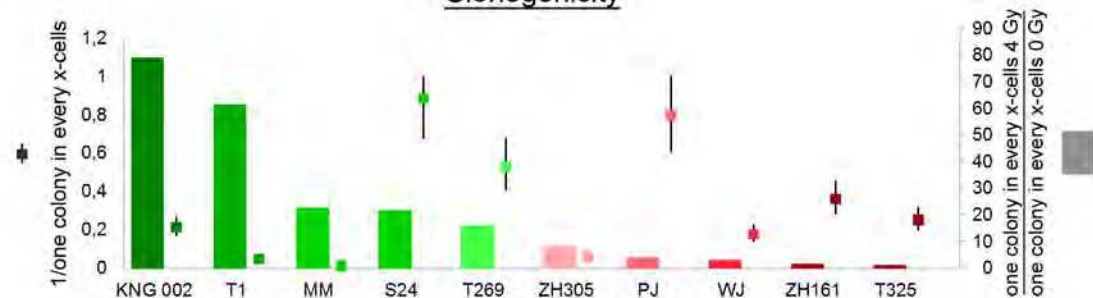
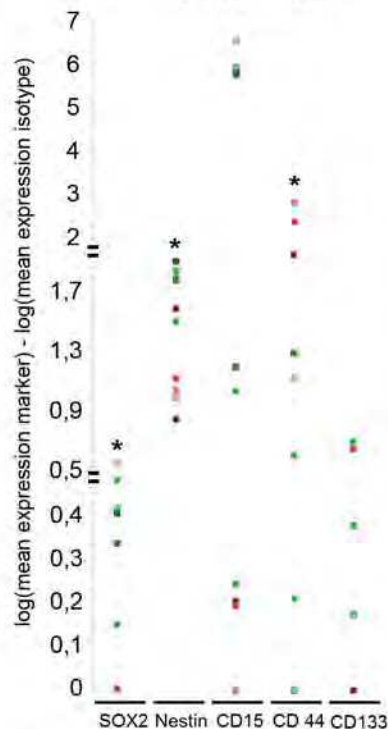


Fig. 1E

Clonogenicity



Flow Cytometry



mRNA

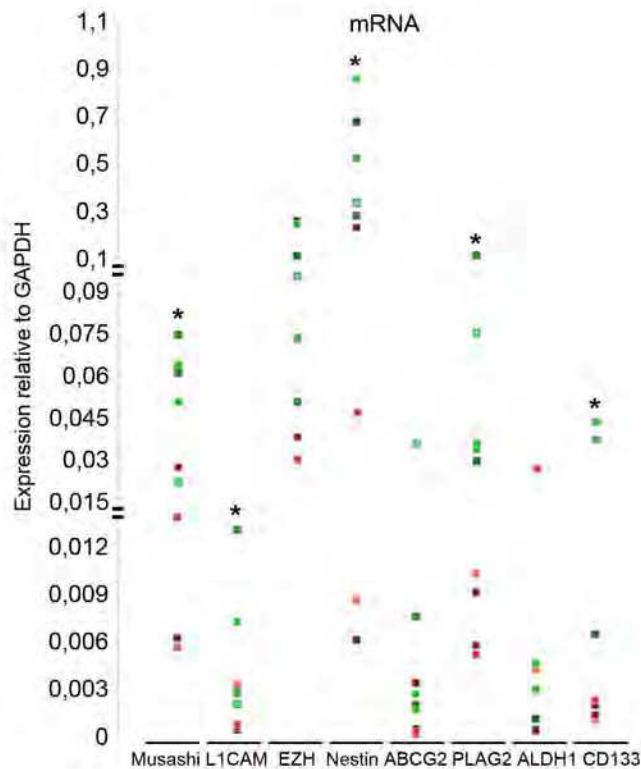
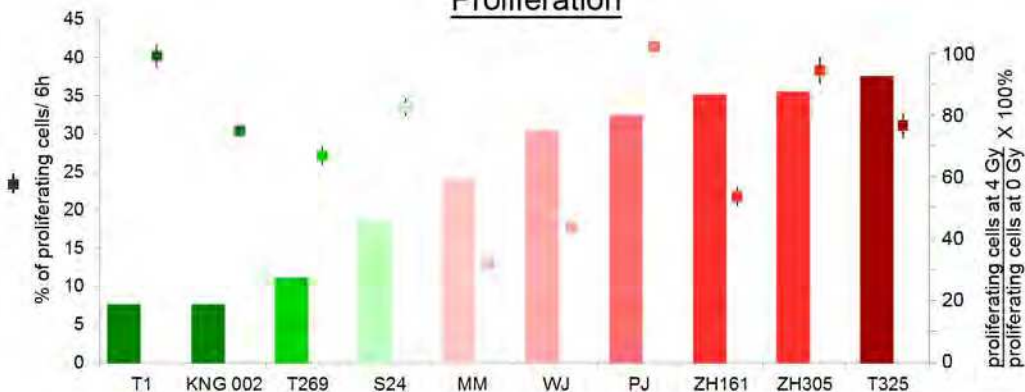
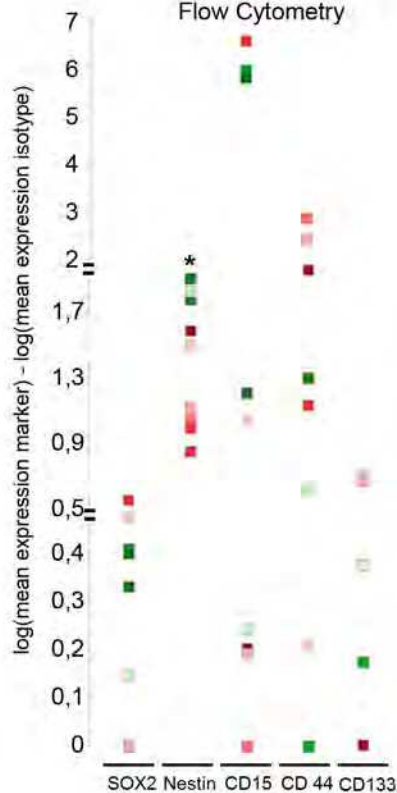


Fig. 2

Proliferation



Flow Cytometry



mRNA

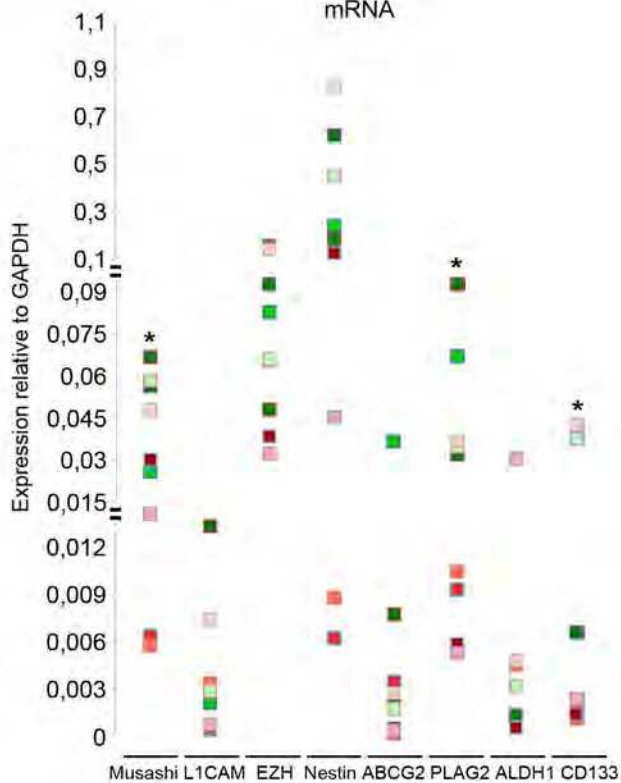
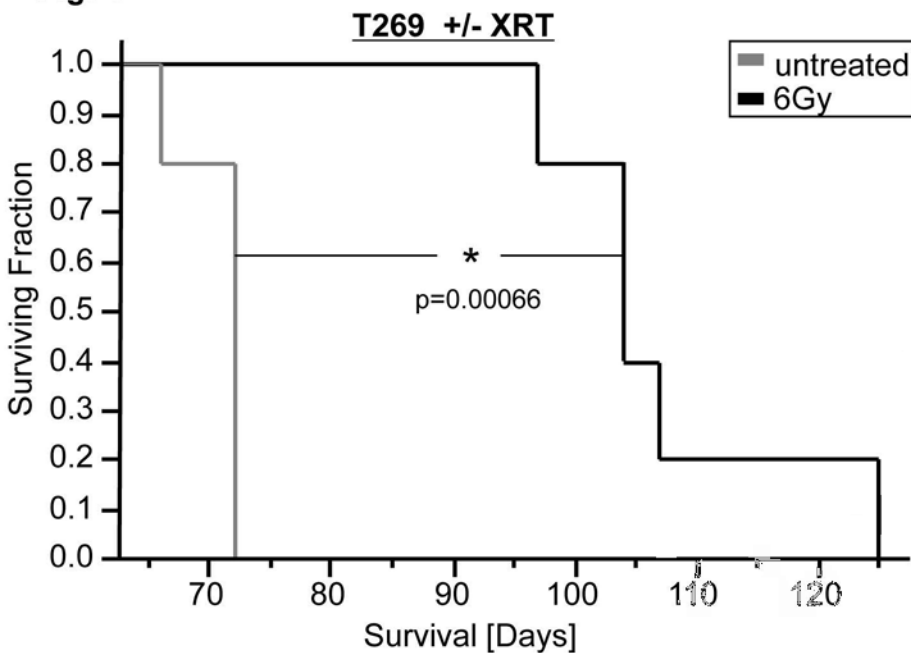
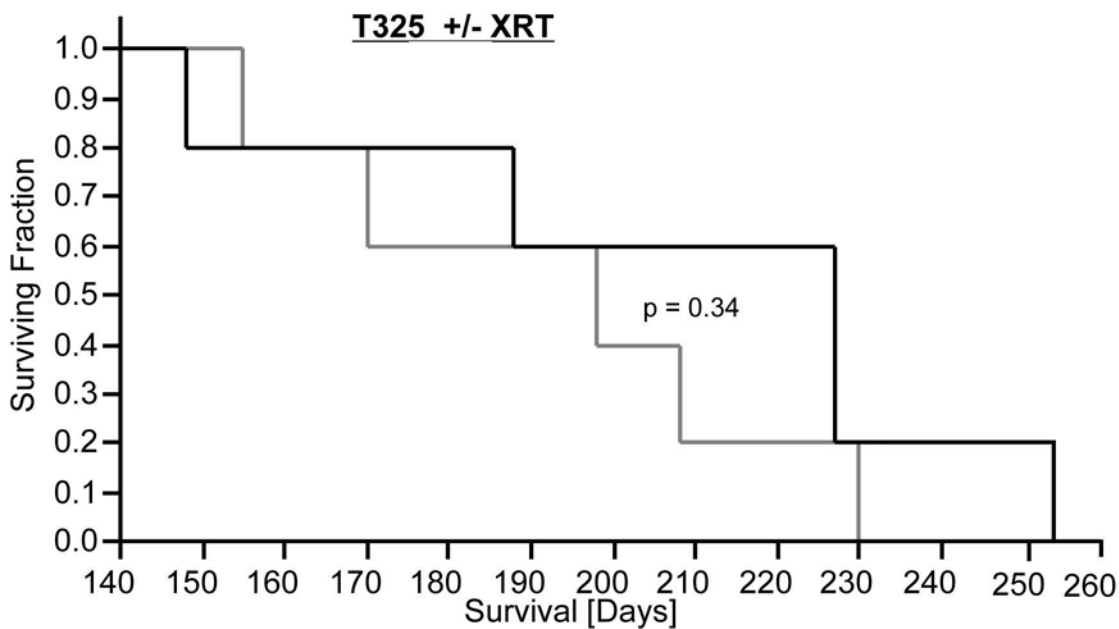


Fig. 3

Fig. 4

| Tumor | 6 Gy | Median survival time (days) |
|-------|------|-----------------------------|
| T269 | - | 71 +/- 2,4 |
| T269 | + | 107 +/- 9,4 |
| T325 | - | 192 +/- 26,8 |
| T325 | + | 220 +/- 47,2 |



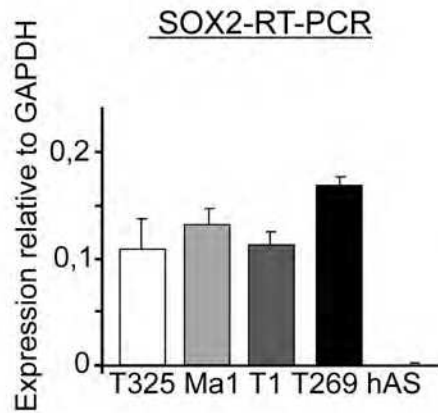


Fig. 5A

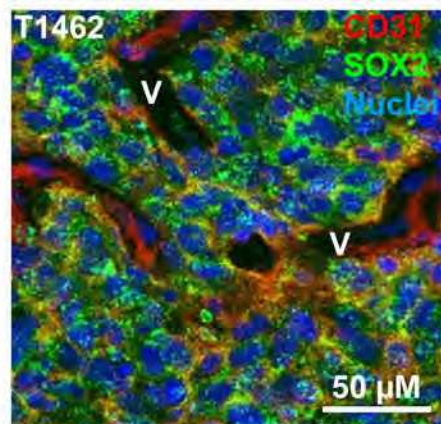


Fig. 5B

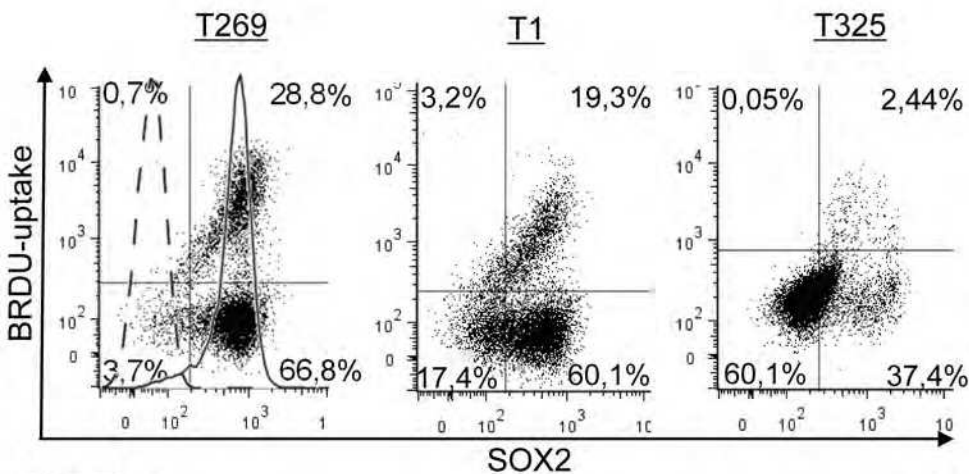


Fig. 5C

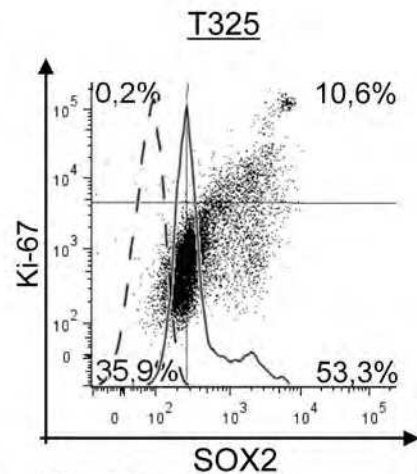


Fig. 5D

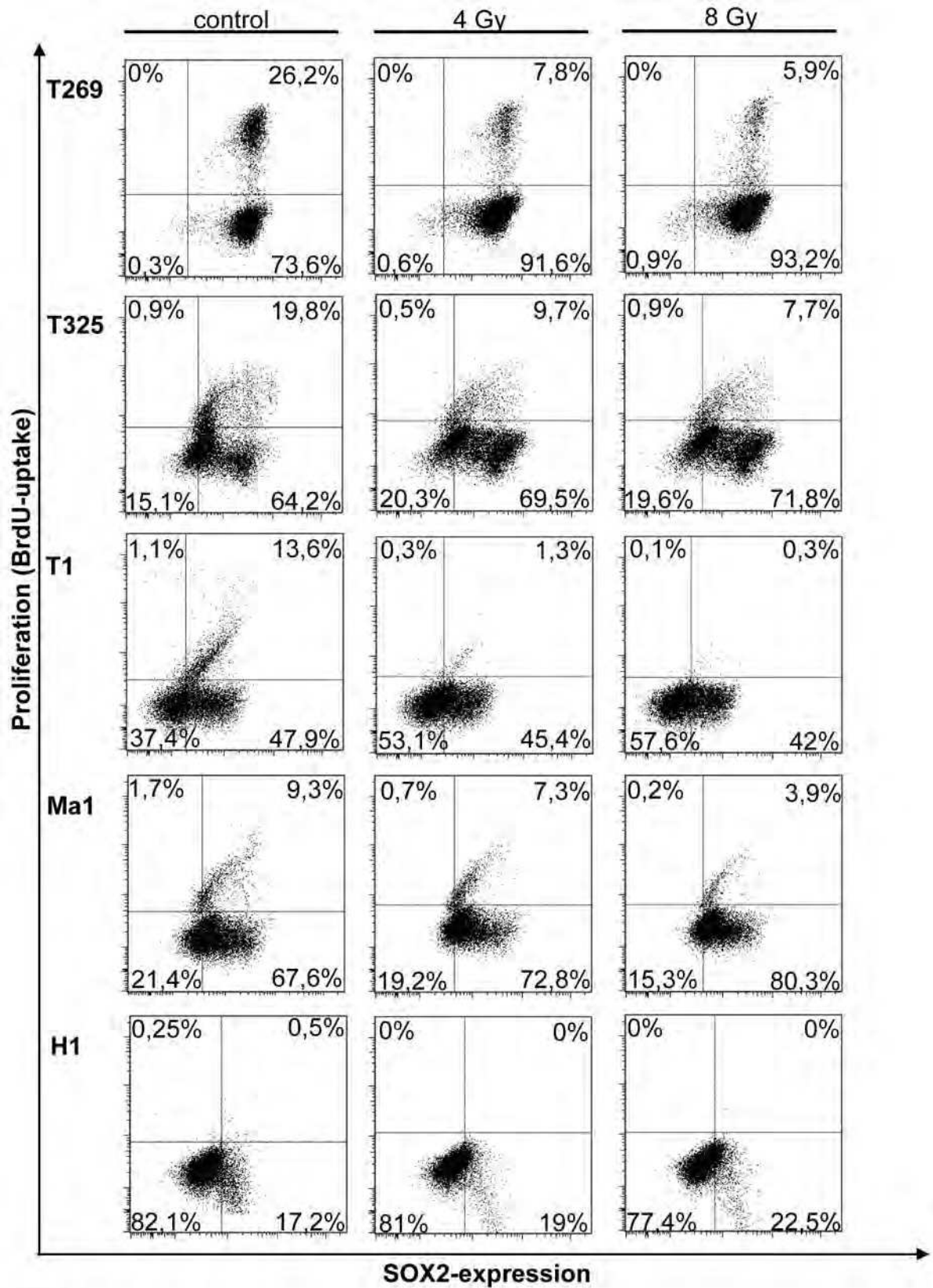


Fig. 6

Supporting information

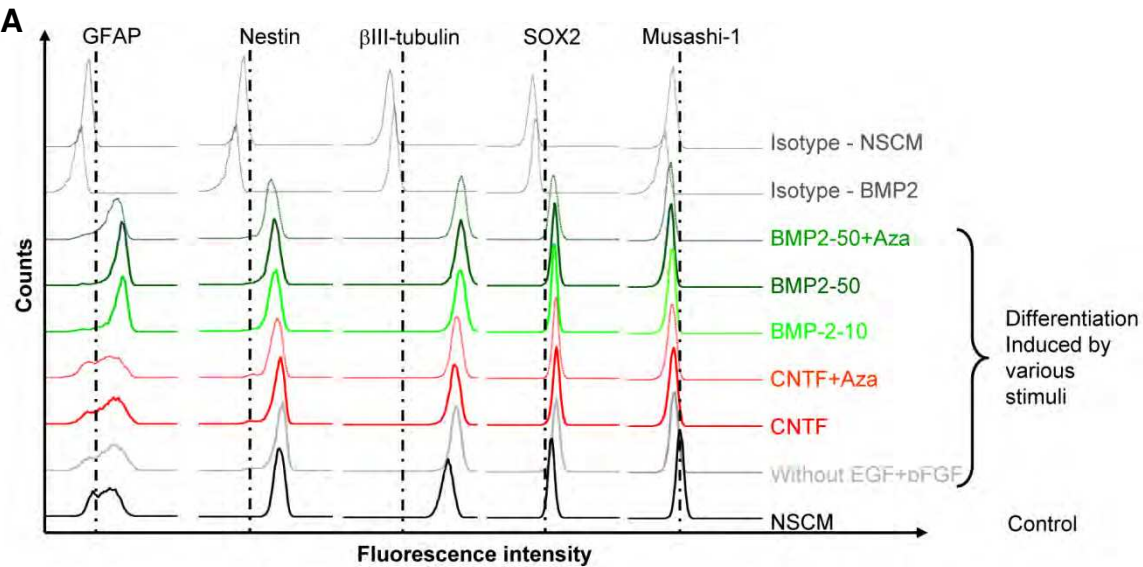
Primary Glioblastoma Cultures:

Can Profiling of Stem Cell Markers Predict Radiotherapy Sensitivity?

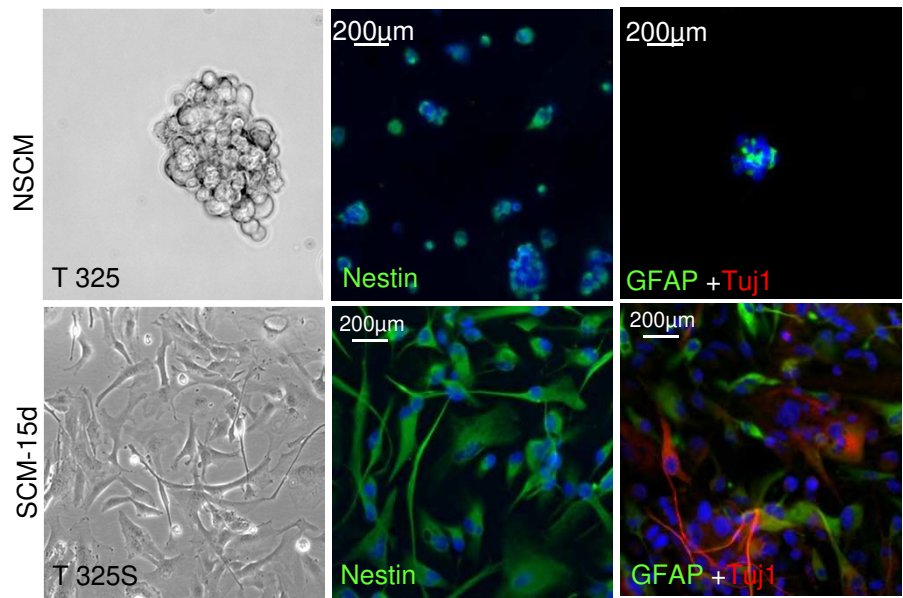
Dieter Lemke^{1,2,3}, Markus Weiler^{1,2,3}, Jonas Blaes^{1,2}, Benedikt Wiestler^{1,2,3}, Leonie Jestaedt⁴, Ann-Catherine Klein^{1,2}, Sarah Löw^{1,2,3}, Günter Eisele¹⁰, Bernhard Radlwimmer^{1,11}, David Capper¹², Kirsten Schmieder⁵, Michel Mittelbronn^{6,7}, Stephanie E. Combs⁹, Martin Bendszus⁴, Michael Weller¹⁰, Michael Platten^{1,3,8}, Wolfgang Wick^{1,2,3*}

¹German Cancer Consortium (DKTK), ²Clinical Cooperation Unit Neurooncology, German Cancer Research Center (DKFZ), Heidelberg, ³Department of Neurooncology, Neurology Clinic and National Center for Tumor Diseases, University of Heidelberg, ⁴Department of Neuroradiology, University of Heidelberg, ⁵Department for Neurosurgery, Universitätsmedizin of Mannheim, ⁶Institute for Brain Research, University of Tübingen, ⁷Institute of Neurology (Edinger Institute), Goethe University, Frankfurt/Main, Germany, ⁸Clinical Cooperation Unit Neuroimmunology and Brain Tumor Immunology, DKFZ Heidelberg, ⁹Department of Radiation Oncology, University of Heidelberg, ¹⁰Department of Neurology, University Hospital Zurich, Switzerland, ¹¹Division of Molecular Genetics, German Cancer Research Center (DKFZ), Heidelberg, Germany, ¹²Institute of Neuropathology, University Clinic Heidelberg, Heidelberg, Germany.

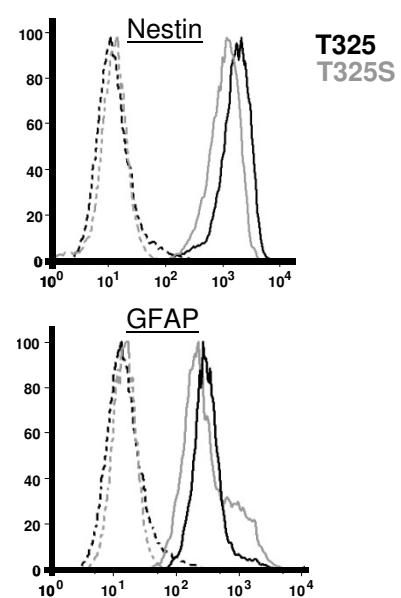
Suppl.Fig. 1A



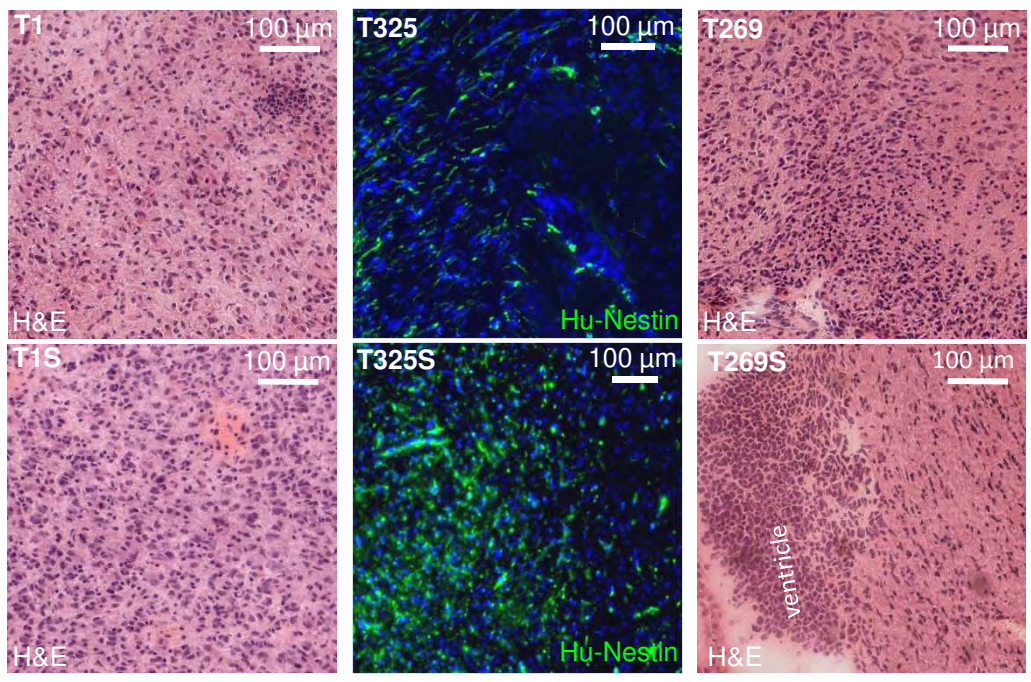
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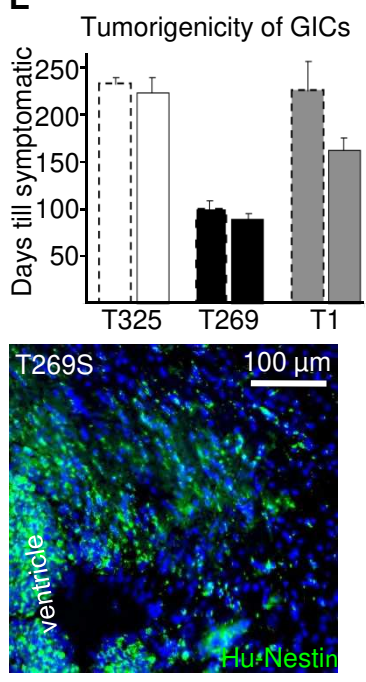
C



D



E



Supplemental Fig. 1:

A: GIC-tumor T269 is multipotent.

Flow cytometric analysis of differentiated markers, GFAP and III-tubulin, as well as the stem cell markers musashi-1, SOX2 and Nestin after induction of differentiation with CNTF, BMP2 (at concentrations of 10 and 50 ng/ml) for 3 days with and without the use of 5-azacytidine or pure growth factor withdrawal. Expression is compared to T269 kept in NSCM. Isotype stainings show that marker staining was specific. GFAP and III-tubulin are upregulated to a various degree depending on differentiation approach. Differentiation approaches do not lower the expression of the stem cell markers examined (dashed line helps to estimate the expression level of the isotype).

B+C: T325 shows multilineage differentiation capacity but cannot be terminally differentiated with the help of serum-containing medium (SCM).

Upper panel:

T325 forms neurospheres in NSCM and shows expression of Nestin and GFAP in immunocytochemistry.

Lower panel:

Differentiation with SCM for 15 (T325S) days leads to formation of a monolayer in T325 with upregulation of GFAP and -III-tubulin expression in immunocytochemistry. Nestin expression is still positive after differentiation with SCM.

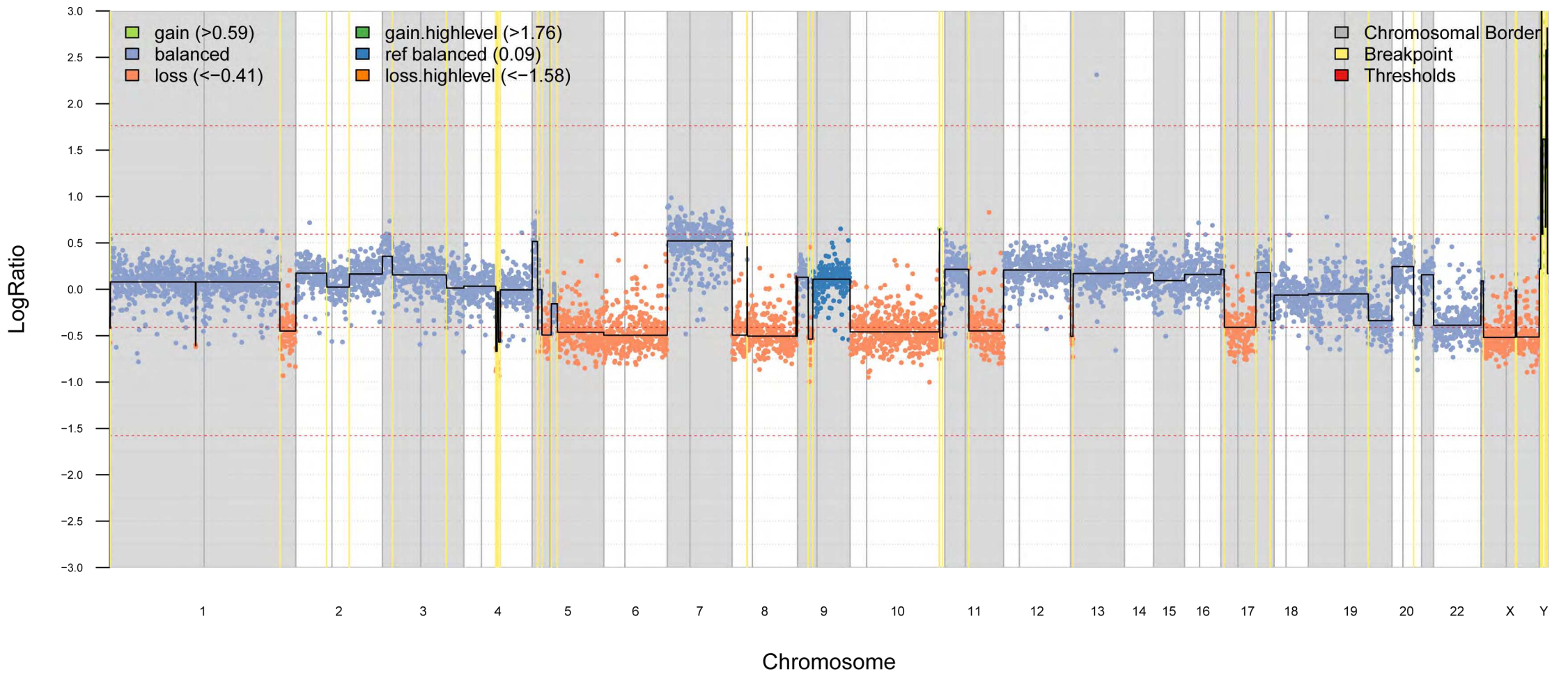
C: Quantification of GFAP and Nestin expression by flow cytometry demonstrates that differentiation of T325 (T325S) for 15 days in SCM reduces Nestin expression only marginally and leads to upregulation of GFAP in some cells. (dotted line: isotype; black (undifferentiated) and grey (differentiated) line: specific staining for Nestin and GFAP.

D+E: Tumors T325, T269 and T1 are highly tumorigenic.

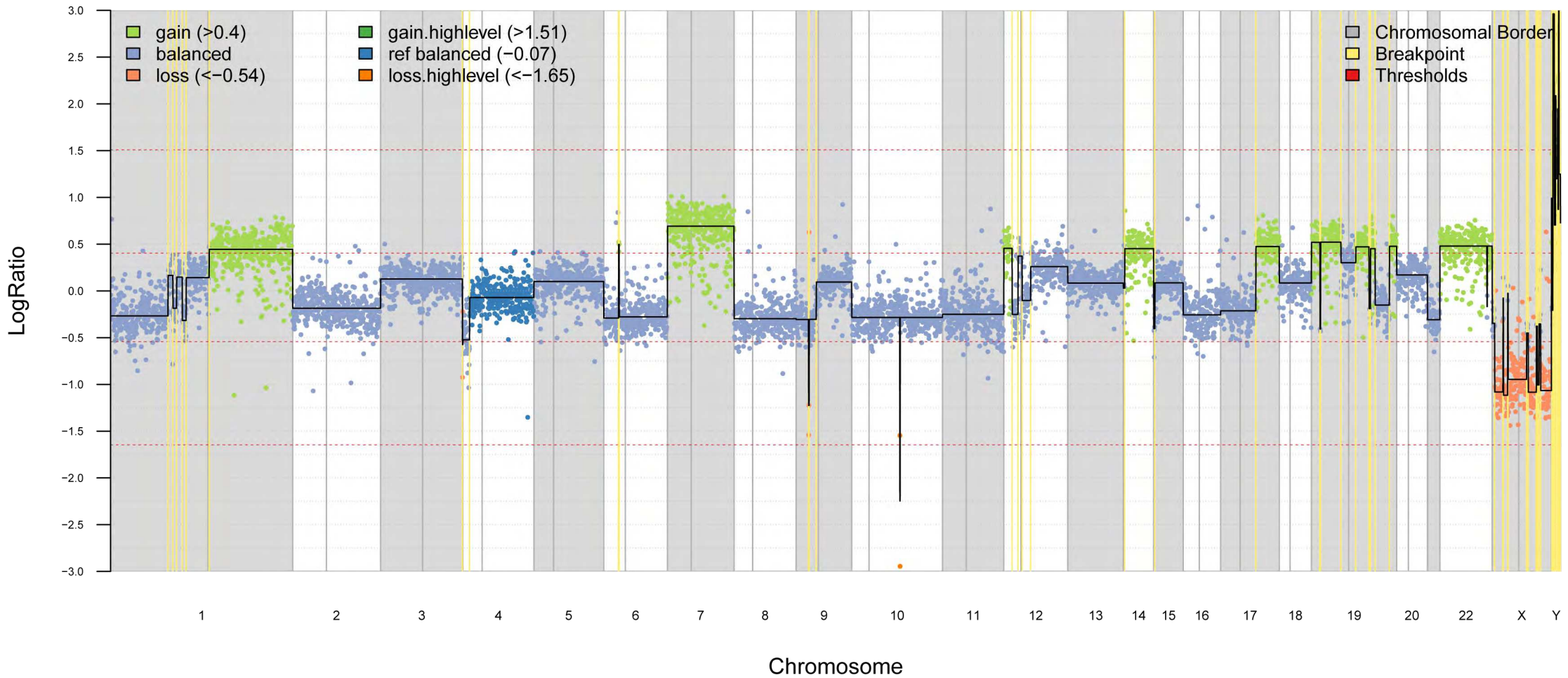
D: Immunofluorescence microscopy against anti-human Nestin and HE (hematoxylin eosin) stainings show that T1, T325 and T269 form tumors in nude mice when kept in NSCM but also after induction of differentiation in SCM for 15 days (T325S, T269S, T1S). Nestin staining shows that cells infiltrate into the surrounding brain.

E: Tumors T325, T269 and T1 form tumors after implantation of only 1000 GIC-culture cells (bars with dotted lines). Generation time of tumors varies from ~90 to more than 200 days in the different GIC-cultures and is faster after implantation of 50.000 cells (bars with dense lines).

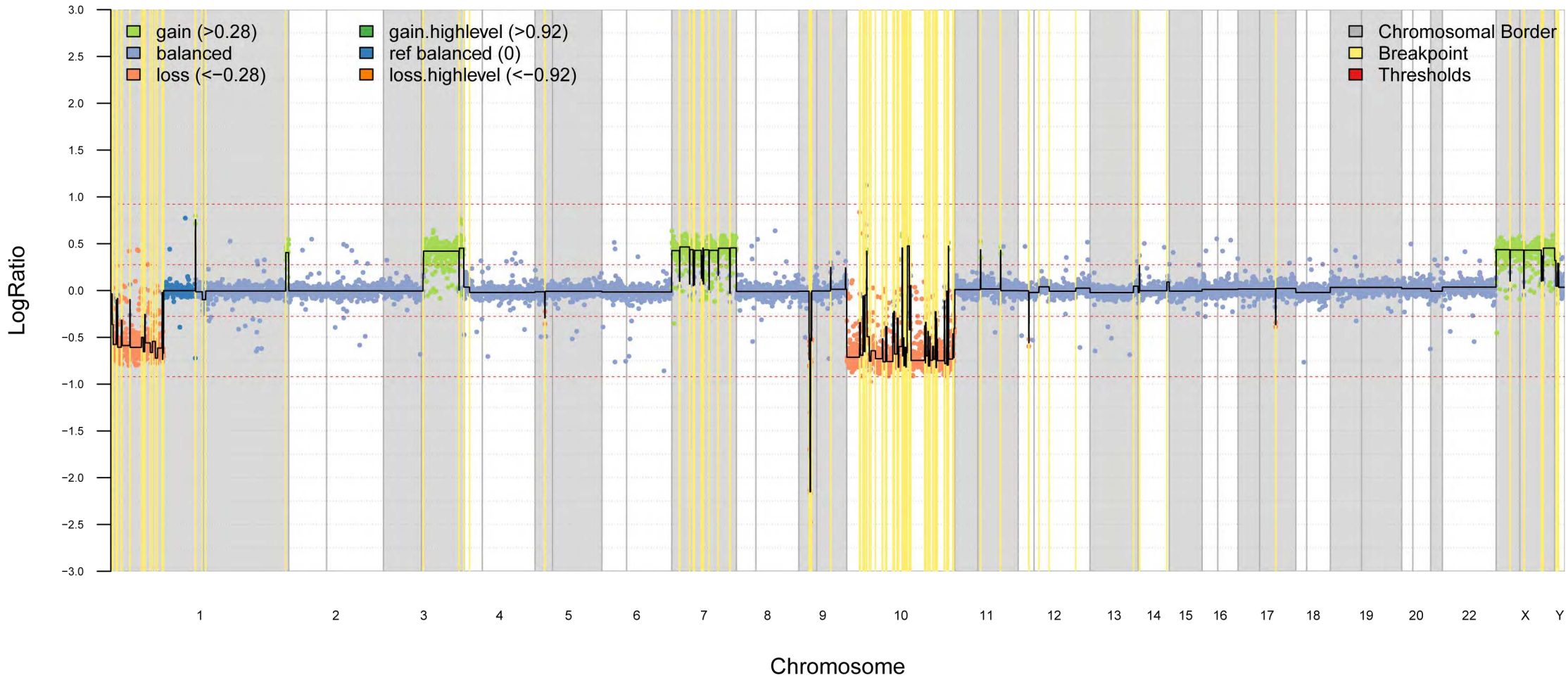
Tumor T325



Tumor T269



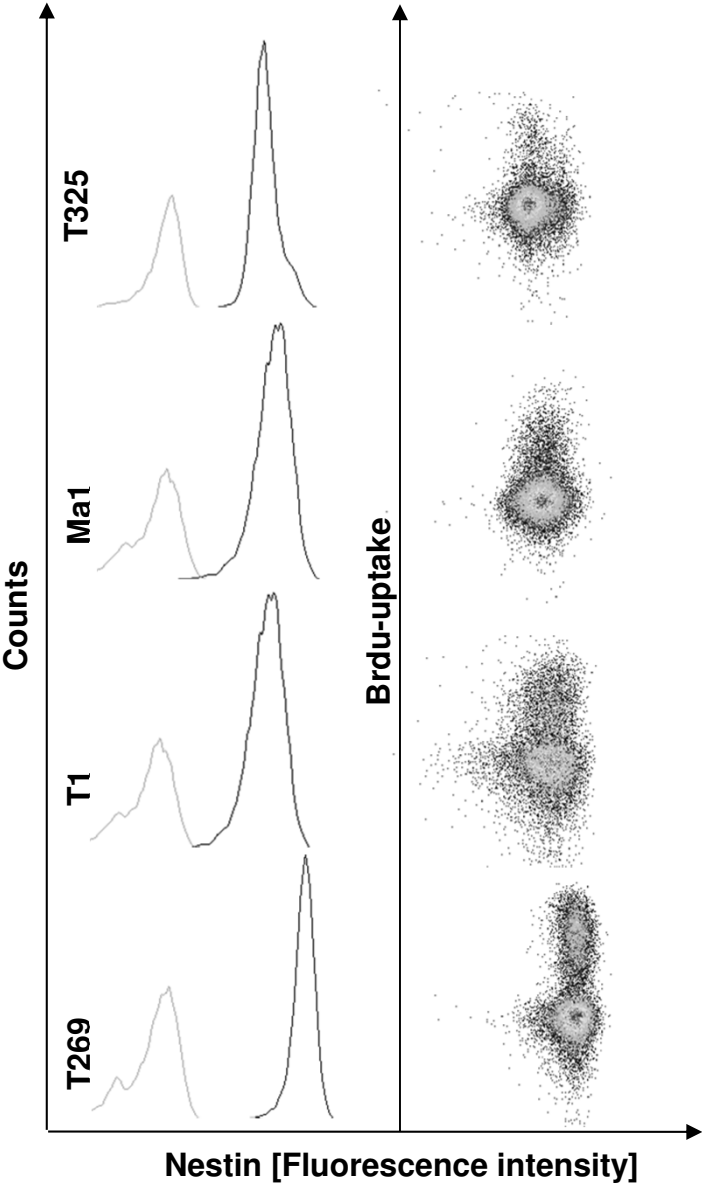
Tumor T1



Supplemental Fig. 2-4:

CGH-analysis (comparative genomic hybridization) of the GIC cultures T325, T1 and T269 underline the glioblastoma origin of the GICs. Losses and gains are visualized and plotted against their origin on the different chromosomes.

Supplemental Fig. 5



Supplemental Fig. 5:

BrdU-uptake was plotted against Nestin expression in 4 different GIC cultures (right side). Cells take up BrdU homogeneously, subfractions with a distinct Nestin expression or proliferation rate can not be detected). Histogramms on the left side show that all the cultures tested are 100 % positive for Nestin and that this marker is normally distributed in the different GIC cultures (black line shows the expression of Nestin, grey line shows isotype expression). Cells were treated for 6 hours with BrdU.